

nibbled producing a ladder of products that are visible when a 3' end label is present on the probe oligonucleotide. When the invader oligonucleotide is omitted from a reaction containing  $Mn^{2+}$ , the probe is nibbled from the 5' end.  $Mg^{2+}$ -based reactions display minimal nibbling of the probe oligonucleotide. In any of these cases, the digestion of the probe is dependent upon the presence of the target nucleic acid. In the examples below, the ladder produced by the enhanced nibbling activity observed in the presence of  $Mn^{2+}$  is used as a positive indicator that the probe oligonucleotide has hybridized to the target sequence.

### EXAMPLE 11

#### Invasive 5' Endonucleolytic Cleavage By Thermostable 5' Nucleases In The Absence of Polymerization

As described in the examples above, 5' nucleases cleave near the junction between single-stranded and base-paired regions in a bifurcated duplex, usually about one base pair into the base-paired region. In this example, it is shown that thermostable 5' nucleases, including those of the present invention (*e.g.*, Cleavase® BN nuclease, Cleavase® A/G nuclease), have the ability to cleave a greater distance into the base paired region when provided with an upstream oligonucleotide bearing a 3' region that is homologous to a 5' region of the subject duplex, as shown in Figure 30.

Figure 30 shows a synthetic oligonucleotide which was designed to fold upon itself which consists of the following sequence: 5'-GTTCTCTGCTCTCTGGTCGCTGTCTCGCTTGTGAAACAAGCGAGACAGCGTGGTCTCTCG-3' (SEQ ID NO:40). This oligonucleotide is referred to as the "S-60 Hairpin." The 15 basepair hairpin formed by this oligonucleotide is further stabilized by a "tri-loop" sequence in the loop end (*i.e.*, three nucleotides form the loop portion of the hairpin) [Hiraro, I. *et al.* (1994) *Nucleic Acids Res.* 22(4):576]. Figure 30 also show the sequence of the P-15 oligonucleotide and the location of the region of complementarity shared by the P-15 and S-60 hairpin oligonucleotides. The sequence of the P-15 oligonucleotide is

5'-CGAGAGACCACGCTG-3' (SEQ ID NO:41). As discussed in detail below, the solid black arrowheads shown in Figure 29 indicate the sites of cleavage of the S-60 hairpin in the absence of the P-15 oligonucleotide and the hollow arrow heads indicate the sites of cleavage in the presence of the P-15 oligonucleotide. The size of the arrow head indicates the relative utilization of a particular site.

The S-60 hairpin molecule was labeled on its 5' end with biotin for subsequent detection. The S-60 hairpin was incubated in the presence of a thermostable 5' nuclease in the presence or the absence of the P-15 oligonucleotide. The presence of the full duplex which can be formed by the S-60 hairpin is demonstrated by cleavage with the Cleavase® BN 5' nuclease, in a primer-independent fashion (*i.e.*, in the absence of the P-15 oligonucleotide). The release of 18 and 19-nucleotide fragments from the 5' end of the S-60 hairpin molecule showed that the cleavage occurred near the junction between the single and double stranded regions when nothing is hybridized to the 3' arm of the S-60 hairpin (Figure 31, lane 2).

The reactions shown in Figure 31 were conducted as follows. Twenty fmole of the 5' biotin-labeled hairpin DNA (SEQ ID NO:40) was combined with 0.1 ng of Cleavase® BN enzyme and 1 µl of 100 mM MOPS (pH 7.5) containing 0.5% each of Tween-20 and NP-40 in a total volume of 9 µl. In the reaction shown in lane 1, the enzyme was omitted and the volume was made up by addition of distilled water (this served as the uncut or no enzyme control). The reaction shown in lane 3 of Figure 31 also included 0.5 pmole of the P15 oligonucleotide (SEQ ID NO:41), which can hybridize to the unpaired 3' arm of the S-60 hairpin (SEQ ID NO:40), as diagrammed in Figure 30.

The reactions were overlaid with a drop of mineral oil, heated to 95°C for 15 seconds, then cooled to 37°C, and the reaction was started by the addition of 1 µl of 10 mM MnCl<sub>2</sub> to each tube. After 5 minutes, the reactions were stopped by the addition of 6 µl of 95% formamide containing 20 mM EDTA and 0.05% marker dyes. Samples were heated to 75°C for 2 minutes immediately before electrophoresis through a 15% acrylamide gel (19:1 cross-linked), with 7 M urea, in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4 mM EDTA.

After electrophoresis, the gel plates were separated allowing the gel to remain flat on one plate. A 0.2 mm-pore positively-charged nylon membrane (NYTRAN, Schleicher and Schuell, Keene, NH), pre-wetted in H<sub>2</sub>O, was laid on top of the exposed gel. All air bubbles were removed. Two pieces of 3MM filter paper (Whatman) were then placed on top of the membrane, the other glass plate was replaced, and the sandwich was clamped with binder clips. Transfer was allowed to proceed overnight. After transfer, the membrane was carefully peeled from the gel and allowed to air dry. After complete drying, the membrane was washed in 1.2X Sequenase Images Blocking Buffer (United States Biochemical) using 0.3 ml of buffer/cm<sup>2</sup> of membrane. The wash was performed for 30 minutes at room temperature. A streptavidin-alkaline phosphatase conjugate (SAAP, United States Biochemical) was added to a 1:4000 dilution directly to the blocking solution, and agitated for 15 minutes. The membrane was rinsed briefly with H<sub>2</sub>O and then washed three times for 5 minutes per wash using 0.5 ml/cm<sup>2</sup> of 1X SAAP buffer (100 mM Tris-HCl, pH 10, 50 mM NaCl) with 0.1% sodium dodecyl sulfate (SDS). The membrane was rinsed briefly with H<sub>2</sub>O between each wash. The membrane was then washed once in 1X SAAP buffer containing 1 mM MgCl<sub>2</sub> without SDS, drained thoroughly and placed in a plastic heat-sealable bag. Using a sterile pipet, 5 mls of CDP-Star™ (Tropix, Bedford, MA) chemiluminescent substrate for alkaline phosphatase were added to the bag and distributed over the entire membrane for 2-3 minutes. The CDP-Star™-treated membrane was exposed to XRP X-ray film (Kodak) for an initial exposure of 10 minutes.

The resulting autoradiograph is shown in Figure 31. In Figure 31, the lane labelled "M" contains the biotinylated P-15 oligonucleotide which served as a marker. The sizes (in nucleotides) of the uncleaved S-60 hairpin (60 nuc; lane 1), the marker (15 nuc; lane "M") and the cleavage products generated by cleavage of the S-60 hairpin in the presence (lane 3) or absence (lane 2) of the P-15 oligonucleotide are indicated.

Because the complementary regions of the S-60 hairpin are located on the same molecule, essentially no lag time should be needed to allow hybridization (*i.e.*, to form